Expression profiling reveals meiotic male germ cell mRNAs that are translationally up- and down-regulated

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Gametes rely heavily on posttranscriptional control mechanisms to regulate their differentiation. In eggs, maternal mRNAs are stored and selectively activated during development. In the male, transcription ceases during spermiogenesis, necessitating the posttranscriptional regulation of many paternal mRNAs required for spermatozoan assembly and function. To date, most of the testicular mRNAs known to be translationally regulated are initially transcribed in postmeiotic cells. Because protein synthesis occurs on polysomes and translationally inactive mRNAs are sequestered as ribonucleoproteins (RNPs), movement of mRNAs between these fractions is indicative of translational up- and down-regulation. Here, we use microarrays to analyze mRNAs in RNPs and polysomes from testis extracts of prepuberal and adult mice to characterize the translation state of individual mRNAs as spermatogenesis proceeds. Consistent with published reports, many of the translationally delayed postmeiotic mRNAs shift from the RNPs into the polysomes, establishing the validity of this approach. In addition, we detect another 742 mouse testicular transcripts that show dramatic shifts between RNPs and polysomes. One subgroup of 35 genes containing the known, translationally delayed *phosphoglycerate kinase 2* (*Pgk2*) is initially transcribed during meiosis and is translated in later-stage cells. Another subgroup of 82 meiotically expressed genes is translationally down-regulated late in spermatogenesis. This high-throughput approach defines the changing translation patterns of populations of genes as male germ cells differentiate and identifies groups of meiotic transcripts that are translationally up- and down-regulated.

microarray | polysome | ribonucleoprotein particles | spermatogenesis | translational control

The testis contains a diverse population of somatic and germ cell types. As spermatogenesis proceeds, diploid spermatogonia differentiate into meiotic spermatocytes, which divide twice without additional DNA replication, producing haploid round spermatids (1, 2). These spermatids transform into highly polarized and uniquely shaped spermatozoa. As the germ cells differentiate, the changing amounts and populations of mRNAs in the germ cells and somatic cells have been well documented by microarray analyses (3–7) and by the cloning and sequencing of cDNA libraries prepared from highly purified populations of individual cell types (8, 9).

Although these microarray and cloning studies provide valuable insight into the temporal appearance/disappearance of individual mRNAs, they do not address the question of when the proteins encoded by the mRNAs are synthesized. In the germ cells of the testis, a temporal disconnect between mRNA transcription and protein synthesis is especially common, in part because RNA synthesis terminates during midspermiogenesis long before the spermatid completes its differentiation into the spermatozoon (1). Thus, posttranscriptional mechanisms play major roles in the temporal regulation of protein synthesis in developing male gametes.

The translation of mRNAs is a determining factor in defining cell and tissue phenotypes. Translationally inactive mRNAs are often defined as those sequestered in ribonucleoprotein (RNP) particles, whereas polysomal mRNAs are usually undergoing active translation. Sucrose gradient fractionation of total adult testis extracts has identified many male germ cell mRNAs that are predominantly in RNPs (1, 10). Some of these mRNAs encoding proteins, such as the protamines, are stored for up to a week as RNPs before being translated (11). To date, most of the known translationally delayed male germ cell mRNAs are first transcribed long after meiosis by the haploid expressed transcription factor cAMP-responsive element modulator tau (12–14).

Microarray studies have provided valuable insights into gene expression patterns in numerous organisms, tissues, and pathological states. Here, we combine sucrose gradient fractionation of prepuberal and adult mouse testis extracts with microarray analyses to define the translation profile of the mouse testis. Monitoring mRNA movement between RNPs and polysomes allows us to examine the mobilization and polysomal release of mRNAs as male germ cells differentiate. By using the shifts of known, translationally delayed mRNAs between RNPs and polysomes as a means to validate this approach, we have identified one group of meiotically expressed mRNAs that are translationally up-regulated and one group of meiotically expressed mRNAs that exhibit translational down-regulation in late-stage germ cells.

Results

Identification of Differentially Expressed Transcripts in Prepuberal and Adult Testes. To identify previously uncharacterized genes that are posttranscriptionally regulated in the mouse testis, we have analyzed expression profiles of mRNAs isolated from RNPs and polysomes as spermatogenesis advances. In the first wave of spermatogenesis after birth, meiotic and postmeiotic cells are present in testes from 17-day-old and 22-day-old mice, respectively, and all cell types are present in adults. Affymetrix (Santa Clara, CA) MOE430A microarray chips containing a total of 22,690 probe sets were used to assess total gene expression. After hybridization, 11,626, 11,630, and 10,751 probe sets were detected with RNA prepared from the testes of 17-day-old mice, 22-day-old mice, and adult mice, respectively. A combined total of 12,229 (54%) different probe sets, including 54 controls, was

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Abbreviations: RNP, ribonucleoprotein; Q-PCR, quantitative RT-PCR; TSN, Translin.

Data deposition: The sequence reported in this paper has been deposited in the Gene Expression Omnibus database (accession no. GSE4711).

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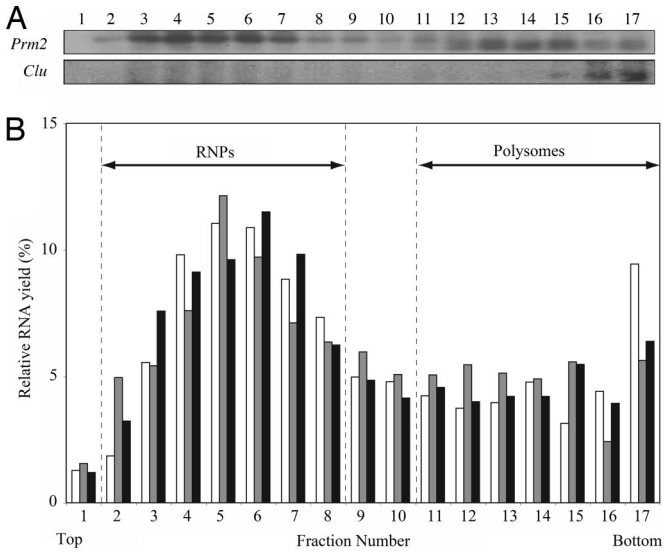

Fig. 1. Profiles of total RNA isolated from fractionated extracts from prepuberal and adult mouse testes. (*A*) Adult testis extracts were fractionated by sucrose gradient centrifugation and aliquots (1/5 volume) of purified RNA were used for Northern blot analysis with probes for *Prm2* and *Clu*. (*B*) Distribution of RNA in the fractionated extracts from prepuberal and adult mouse testes. Open bars indicate RNA from testes of 17-day-old mice, gray bars indicate RNA from testes of 22-day-old mice, and black bars indicate RNA from testes of adult mice. The gradient bottom is to the right.

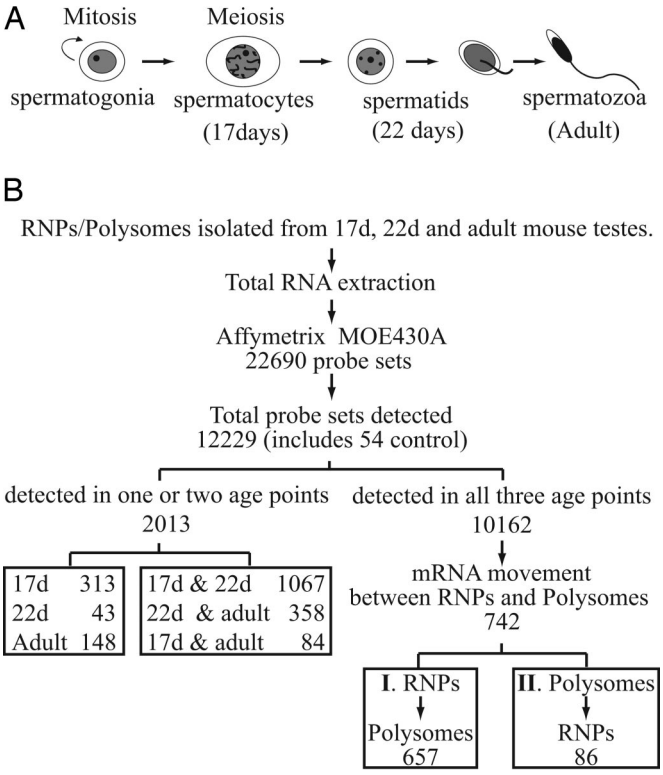

Fig. 2. Schematic summary of fractionation protocol used to examine the mobilization of mRNAs between RNPs and polysomes. This flow diagram shows the temporal pattern of germ cell differentiation in the first wave of spermatogenesis (*A*) and the number of probe sets detected in RNAs isolated from total, RNP, and polysomal fractions from testes of prepuberal and adult mice (*B*). 17d, 17-day-old; 22d, 22-day-old.

detected from the three developmental stages (Table 3, which is published as supporting information on the PNAS web site).

As predicted from the published expression profiling studies for which developing testes and individual testicular cell types were investigated (5, 6), many transcripts are only detected at a particular stage of testicular development, often an indication of transcription in a newly differentiating cell type. In testes from 17-day-old mice, 22-day-old mice, and adult mice, 313, 43, and 148 stage-enriched probe sets were detected (Tables 4–6, which are published as supporting information on the PNAS web site). These findings are in agreement with the published temporal expression patterns of testicular mRNAs (5).

To confirm the validity of our approach, we have compared our data to known databases for mRNAs known to be present in the testes of 22-day-old and adult mice but absent in the testes of 17-day-old mice (Table 7, which is published as supporting information on the PNAS web site). The transition proteins (*Tnp*) 1 and 2, the protamines (*Prm*) 1 and 2, *Hils1*, and *Akap4*, six mRNAs known to be initially transcribed in postmeiotic cells, were among the 358 probe sets detected (10, 15–17). Other mRNAs, known to be expressed during meiosis, such as *Clgn* and *Msh4* (Table 3), were detected in the testes of 17-day-old mice (18, 19).

To assess the translational status of mRNAs, sucrose gradient centrifugation of total testis extracts from prepuberal and adult mice was performed to isolate RNPs and polysomes enriched for meiotic (17-day-old mice) or postmeiotic (22-day-old mice and adult mice) populations of mRNAs. To confirm the separation of RNPs and polysomal mRNAs and calibrate the gradients, Northern blot analysis was performed with probes for *Prm2* and clusterin (*Clu*) (Fig. 1*A*). After their transcription in haploid germ cells, *Prm2* mRNAs are stored in RNPs with poly(A) tails of ≈160 nt and move onto polysomes for translation ≈1 week later with a shortened poly(A) tail (11). *Clu* mRNA, expressed in Sertoli cells, is not translationally regulated, and its mRNAs are primarily on polysomes. As expected, the longer stored form of *Prm2* mRNAs was predominantly in RNPs (fractions 3–7), whereas the partially deadenylated *Prm2* mRNAs were in polysomes (fractions 13–15) (10). The presence of *Clu* mRNA in polysomes (fractions 15–17), but not in RNPs, establishes the integrity of the polysomes in the fractionated extracts. Similar amounts of purified RNAs were obtained from RNPs and polysomes prepared from the testes of 17-day-old mice, 22-day-old mice, and adult mice in three separate experiments with three different extracts (Fig. 1*B*). Based on the predicted fractionation of *Prm2* and *Clu* in the RNPs and polysomes, tubes 2–8 and 11–17 were pooled and their RNAs were used as RNP and polysome templates, respectively, for expression profiling.

Microarray Analysis of RNP and Polysomal mRNAs Detects the Predicted Shifts in Translationally Delayed Postmeiotic mRNAs. Assuming that actively translated mRNAs are in polysomes and translationally inactive mRNAs are in RNPs, we have arbitrarily defined movement of mRNAs between RNPs and polysomes (up-regulation or down-regulation) as a redistribution of at least 20% between fractions. To demonstrate that mRNA movement of this magnitude represents physiologically meaningful changes, we have focused on the redistribution of a number of known translationally delayed postmeiotically expressed mRNAs in prepuberal and adult testes (Fig. 2). As expected, the *Prm2*, *Tnp1*, *Hils1*, *Aif1*, and *Act* marker mRNAs are among the 112 of 358 probe sets (31%) whose mRNAs move from RNPs in 22-day-old mice onto polysomes in the adult (Table 7) (1, 14, 20, 21). This distribution demonstrates that the subcellular fractionation and the 20% change ($P < 0.05$) we use to assess functional movement of mRNAs between RNPs and polysomes detects shifts of translationally regulated germ cell mRNAs and will

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allow us to discover new genes that are translationally up- or down-regulated.

To confirm the validity of the microarray changes, quantitative RT-PCR (Q-PCR) assays were carried out for 10 representative genes [four prepuberally enriched (*Igf2*, *Ccnt2*, *Ets1*, and *Dnmt3b*), two meiotically expressed (*Pgk2* and *Smcp*), and four postmeiotically enriched (*Adam1a*, *Klk21*, *Prm2*, and *Hils1*)]. The relative amounts of mRNAs detected in the Q-PCR assays correlate well with the amounts of mRNAs detected by the microarrays (Table 8, which is published as supporting information on the PNAS web site). Similarly, assays of several RNP-associated mRNAs show good correlations between the microarrays and Q-PCR assays (Table 9, which is published as supporting information on the PNAS web site).

Many mRNAs That Are Transcribed During Meiosis Move Between RNPs and Polysomes. In mammalian male germ cells, there are dramatic shifts in the mRNA distribution between RNPs and polysomes as they differentiate into mature gametes (10). Analyzing the mRNAs that showed at least 20% transcript redistribution between polysomes and RNPs ($P < 0.05$), 742 (668 UniGene clusters, and 5 probe sets that were not clustered) were identified. These mRNAs were divided into two groups: group I, which contains 657 probe sets (587 UniGene clusters and 5 unmapped probes) whose mRNAs move onto polysomes with increasing mouse age, and group II, which contains 86 probe sets (82 UniGene clusters) that redistribute from polysomes to RNPs (Fig. 2; see also Fig. 3, which is published as supporting information on the PNAS web site). One gene, *allantoicase* (*Allc*) (number 76 in Table 10, which is published as supporting information on the PNAS web site) is present in both groups I and II, because its mRNA moves onto RNPs in 22-day-old mice and back onto polysomes in adult mice.

Translationally Up-Regulated Meiotic mRNAs. We have subdivided the mRNAs that are translationally up-regulated with germ cell development into three subgroups (Groups Ia, 17-day-old to 22-day-old; Ib, 22-day-old to adult; and Ic, 17-day-old to adult). Group Ia contains 65 probe sets, Group Ib contains 85 probe sets, and Group Ic contains 507 probe sets whose mRNAs redistribute from RNPs to polysomes (Table 10).

Although many mRNAs first expressed in haploid cells show dramatic movement onto polysomes as spermatogenesis advances (1, 22), the *Pgk2* mRNA is one of the few mRNAs transcribed during meiosis and known to be translationally regulated after meiosis (23). *Pgk2* mRNAs are sequestered as RNPs in pachytene spermatocytes and, with continued transcription, move onto polysomes in later stage spermatids (11, 23–25). By using *Pgk2* as a marker mRNA to detect populations of mRNAs showing similar meiotic expression and translational delays, >70% of *Pgk2* mRNA fractionates as RNPs in the testes of 17-day-old and 22-day-old mice and redistributes onto polysomes in adult mice (Table 10). Fourteen additional mRNAs show similar RNP and polysome distributions (Table 1) (26–36), all of which meet the strict criterion of at least 70% in RNPs in prepuberal testes and show a shift of 20% or more to polysomes in adults. Four genes can be added to this group when we lower the percentage of mRNA in the RNPs of 22-day-old mice from 70% to 62% (Table 1). Relaxing our percentage difference between RNPs and polysomes to 15%, we detect 16 additional meiotic transcripts with a similar mobilization pattern as *Pgk2* (Table 1).

Because the testis is a multicellular organ, it is important to establish that the 15 polysomal up-regulated mRNAs are present in the germ cells of the testis. *Testis-expressed gene 27* (*Tex27*), *spermatogenesis associated 6* (*Spata6*), *outer dense fiber of sperm tails 2* (*Odf2*), *diazepam binding inhibitor-like 5* (*Dbil5*), and *Pgk2* are known to be expressed in male germ cells (25–28, 36). The

Table 1. Meiotic mRNAs showing *Pgk2*-like movement from RNPs to polysomes

Gene	Biologcal process and molecular function
*Tex27**	Transcription, Zn finger, spermatogenesis
*Spata6**	Motor domain, spermatogenesis
*Sh3glb1**	Apoptosis, lipid binding, transferase, mitochondrial morphology
*Ptdss2**	Lipid metabolism, transferase
*Pon2**	Lipid binding, hydrolase, oxidative stress
*Pgk2**	Kinase, glycolysis, spermatogenesis
*Odf2**	Cytoskeleton, spermatogenesis
*Gfer**	Sulphydryl oxidase, spermatogenesis
*Dp1l1**	Intracellular trafficking, receptor
*Ddc8**	Spermatogenesis
*Dbil5**	Lipid binding, steroidogenesis, spermatogenesis
*Dact1**	Antagonist of β-catenin
*Ankrd5**	Transcription, DNA binding, ion binding
*Hdhd1a**	Hydrolase
*2210409M21Rik**	Protein prenylation, transferase
Rnf103†	Nucleic acid binding, ubiquitination, Zn finger
Gk-rs1†	Kinase, oxidoreductase, transferase
Phgdhl1†	Dehydrogenase
4933434I20Rik†	—
Meal‡	Cell differentiation, development, spermatogenesis
Tcp10‡	ATP binding, transferase, spermatogenesis
Serf1‡	Modifier of spinal muscular atrophy candidate
Pcmt1‡	Protein amino acid methylation, transferase
Sdh1‡	Metal ion binding, oxidoreductase, NAD-binding
Ftl1‡	Metal ion binding, iron ion transport
Stard6‡	Steroidogenesis, lipid transport, lipid binding
Rpgrip1‡	Axoneme, sensory perception, cell development
Crat‡	Carnitine *O*-acetyltransferase, transport
Rbed1‡	Nucleic-acid-binding
Spag6‡	Cytoskeleton, spermatogenesis, motility
Cct6b‡	ATP binding, protein folding, chaperone
Dkkl1‡	Signal transduction
4933415F23Rik‡	Protein phosphatase-1 inhibitor-like
1700010I14Rik‡	—
4930547C10Rik‡	—

—, Biological process or molecular function is unknown.
*Transcripts with at least 70% in RNPs in 22-day-old mouse testes and a shift of at least 20% to polysomes in adults.
†Transcripts with at least 60% in RNPs in 22-day-old mouse testes and a shift of at least 20% to polysomes in adults.
‡Transcripts with at least 60% in RNPs in 22-day-old mouse testes and a shift of at least 15% to polysomes in adults.

Q-PCR assays confirm that the remaining genes [*SH3-domain GRB2-like B1* (*Sh3glb1*), *phosphatidylserine synthase 2* (*Ptdss2*), *paraoxonase 2* (*Pon2*), *growth factor*, *erv1-like* (*Gfer*), *deleted in polyposis 1-like 1* (*Dp1l1*), *Ddc8, dapper homolog 1* (*Dact1*), *ankyrin repeat domain 5* (*Ankrd5*), the EST *2210409M21*, and *haloacid dehalogenase-like hydrolase domain 1a* (*Hdhd1a*)] are also more highly expressed in wild-type testes than $Kit^{w}/Kit^{w\text{-}v}$ testes (testes from $Kit^{w}/Kit^{w\text{-}v}$ mice contain somatic cells and spermatogonia, but lack later stage germ cells), establishing their expression in germ cells (data not shown). Based on these assays and their temporal expression, the 14 germ cell mRNAs in the *Pgk2* group represent a group of target mRNAs that move from RNPs to polysomes in male meiotic germ cells.

Translationally Down-Regulated Meiotic mRNAs. Although RNP/polysome studies generally focus on the mobilization of mRNAs, we also have detected a group of mRNAs whose distribution shifts from polysomes to RNPs as spermatogenesis proceeds

Table 2. Cluster analysis of Group I (up-regulated) and Group II (down-regulated) genes

Category	Group I	Group II
Cellular metabolism	229 (34.9)	39 (45.3)
Catalytic activity	180 (27.4)	32 (37.2)
Cell growth and/or maintenance	149 (22.7)	28 (32.6)
Protein binding	118 (18.0)	18 (20.9)
Protein metabolism	97 (14.8)	17 (19.8)
Purine nucleotide binding	70 (10.7)	11 (12.8)
Hydrolase activity	69 (10.5)	14 (16.3)
DNA binding	66 (10.0)	12 (14.0)
Development	65 (9.9)	0 (0.0)
Transferase activity	65 (9.9)	11 (12.8)
Protein modification	50 (7.6)	13 (15.1)
Intracellular signaling cascade	42 (6.4)	0 (0.0)
Cell proliferation	41 (6.2)	13 (15.1)
Transcription regulator activity	37 (5.6)	7 (8.1)
Kinase activity	34 (5.5)	8 (9.3)
Cell cycle	33 (5.0)	10 (11.6)
Intracellular transport	31 (4.7)	6 (7.0)
RNA binding	32 (4.9)	0 (0.0)
Protein localization	29 (4.4)	0 (0.0)
Cytoskeleton organization and biogenesis	24 (3.7)	5 (5.8)
Lipid metabolism	22 (3.3)	0 (0.0)
Apoptosis	20 (3.0)	0 (0.0)
Ligase activity	19 (2.9)	0 (0.0)
Cytoskeletal protein binding	16 (2.4)	4 (4.7)
Reproduction	15 (2.3)	0 (0.0)
Biological process not annotated	245 (37.3)	8 (9.3)
Molecular function not annotated	218 (33.2)	3 (3.5)
Total probe sets	657	86

The values in parentheses represent percentages. Proteins with multiple functions or involvement in several biological processes were counted in multiple annotation categories. The genes were annotated by Gene Ontology biological process and Gene Ontology molecular function using DAVID 2.1. The annotation was performed with these settings: threshold of minimum gene counts, 2; maximum *P* value, 0.1.

(Groups IIa–IIc) (Fig. 2 and Table 10). Among a total of 86 probe sets, 37, 23, and 26 probe sets of mRNAs redistribute from polysomes to RNPs between 17-day-old and 22-day-old mice, between 22-day-old mice and adult mice, and between 17-day-old mice and adult mice, respectively.

Cluster analyses comparing the groups of meiotic mRNAs that are up- or down-regulated reveals interesting similarities and differences (Table 2). By using the Gene Ontology annotation, we see that many of the mRNAs in both groups encode cell metabolism and cell growth proteins. However, mRNAs that cluster in development, intracellular signaling, RNA-binding, protein localization, apoptosis, ligase activity, and reproduction are highly enriched in the up-regulated mRNA population. Messenger RNAs encoding proteins with constitutive functions, such as protein modification, cell proliferation, kinase activity, and the cell cycle, are enriched in the down-regulated mRNA population (Table 2). Thus, starting in meiosis, distinct populations of mRNAs are differentially translationally up- or down-regulated in the maturing male germ cell.

Discussion

Expression profiling of testicular mRNAs has defined the expression patterns of many genes essential for the formation of normal sperm and provides valuable insights into the temporal regulation of steady-state gene expression during spermatogenesis (5, 6). In most somatic tissues, transcription and translation are closely linked, making detection of an mRNA a strong indicator of its temporal pattern of protein synthesis. However, both male and female germ cells rely heavily on posttranscriptional regulatory mechanisms. Thus, an initial estimate of the time a protein is likely to be synthesized can be made when mRNAs associate with ribosomes.

Here, we use expression profiling of individual RNP and polysomal mRNAs to examine when proteins are synthesized as germ cells progress through spermatogenesis. We believe that our RNP and polysomal fractions accurately distinguish between stored mRNAs and mRNAs undergoing translation based on the correct fractionation of postmeiotically expressed genes (protamines, transition proteins, *Akap4*, *Hils1*, and *Aif1*) that are known to be translationally controlled (1, 14–16, 20). By using *Pgk2* as a representative meiotic gene undergoing a translational delay (10, 25), this approach has allowed us to identify 34 additional genes with a similar RNP fractionation pattern (23, 24). Seven of the 14 genes showing movement onto polysomes (*Tex27*, *Spata6*, *Odf2*, *Ptdss2*, *Ddc8*, *Dbil5*, and *Hdhd1a*) are highly enriched in testis. Genes, such as *Dbil5*, are under translational control (36) similar to *Pgk2* (25), whereas others, such as *Tex27* (26) and *Spata6* (27), show similar increases in RNA levels in postmeiotic cells. Interestingly, all of the 35 genes are located on autosomes, possibly because of the transcriptional silencing of the sex chromosomes during the pachytene stage of meiotic prophase (37, 38).

In our analyses, we have set strict fractionation criteria requiring >70% of the total mRNA to be in either RNPs or polysomes and analyzing shifts (20% or more) between RNPs and polysomes. Although we have operationally defined all polysomal mRNAs as actively translated mRNAs, we cannot exclude situations when changes in metabolic states induce delayed translation of polysome-bound mRNAs or when mRNAs are in RNPs because of poor translational efficiencies (22) or reduced rates of transcription and/or increased mRNA degradation. Despite such considerations, the correct RNP/polysomal distribution of many mRNAs known to be translationally regulated provides compelling support for the validity of this approach and our experimental techniques.

The Q-PCR has been used to confirm the changing RNP and polysome distributions of the identified meiotic mRNAs. The majority of *Pgk2* and *Dbil5* mRNAs was detected in RNPs in prepuberal mice and in polysomes in adults, consistent with their delayed protein expression (23, 36). In addition, mRNAs, such as *Prm2* and *Smcp*, were not detectable in 17-day-old mice, were primarily in RNPs in 22-day-old mice, and moved onto polysomes in adults (Table 7) (11, 39).

As has been reported (5), the total number of transcripts expressed in prepuberal and adult testes does not vary greatly. Despite significant differential expression of genes in spermatogenic cells, we find that RNA hybridizes to approximately half of the 22,690 probe sets in the Affymetrix microarray chips. Shima *et al.* (5) analyzed testicular RNA from mice 0–56 days postpartum and reported that 29–37% of the transcripts hybridize to Affymetrix microarray chips containing ≈36,000 probe sets. It is likely that these findings reflect differences between the microarray chips used.

We have identified 144 up-regulated UniGene clusters that are exclusively expressed in adult testes but not in the testes of 22-day-old mice. Messenger RNAs encoding proteins, such as the kinesin, KIF17b, carboxylesterase 3, and CD46 antigen, are in this group. KIF17b serves as a molecular motor component of a Translin (TSN)–RNA complex transporting mRNAs transcribed by cAMP-responsive element modulator tau in haploid spermatids from the nucleus to the cytoplasm and through intercellular bridges (12). Because KIF17b has been reported to regulate the intracellular location of the transcriptional coactivator of cAMP-responsive element modulator tau in male germ cells (21), KIF17b links the processes of transcription and transport of mRNAs in the testis. Carboxylesterase accumulates

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in several compartments of the male reproductive tract and plays multiple roles, including the protection of testicular cells from environmental factors (40). The complement regulator membrane cofactor protein, CD46, appears to regulate the sperm acrosome reaction by stabilizing the acrosomal membrane and facilitates sperm–egg interactions (41).

In addition to the 657 probe sets whose mRNAs are up-regulated (Group I) (Table 10), this study has identified a special population of mRNAs whose expression is down-regulated. Eighty-six probe sets encode meiotically expressed mRNAs, such as those encoding the regulatory proteins *Hmgb1* and *Hdac5* (42, 43), which move from polysomes to RNPs, suggesting a decrease in protein synthesis. The mechanism(s) whereby these mRNAs are released from polysomes is of considerable interest because, unlike somatic cells, most nontranslated germ cell mRNAs are not rapidly degraded. Instead, the translationally inactive mRNAs are sequestered in the cytoplasm in a still-translatable form (44). Toward the end of spermatogenesis the cytoplasm is pinched off as residual bodies that are subsequently phagocytized by Sertoli cells (44). Thus, we have identified a population of functionally normal but translationally inactivated mRNAs that reside in the cytoplasm of late-stage germ cells. Krawetz and colleagues (45) recently reported that ejaculated spermatozoa contain a complex repertoire of mRNAs and proposed that some of the mRNAs may be involved in embryo development. Among the 82 meiotically expressed genes that become translationally inactivated with increasing mouse age (Group II) (Table 10), we did not detect any enrichment in the limited number of genes in the embryogenesis or development categories (Table 1). The evolutionary advantages and mechanisms regulating this unique system to down-regulate protein synthesis in the late-stage male germ cells merit further investigation.

Analyzing RNP and polysomal mRNAs by gene profiling to assess the translational state of individual mRNAs is widely applicable to tissues and pathological states in which translational regulation occurs. Approximately 5% of the probe sets in the up-regulated Group I (none from the down-regulated Group II) are classified as RNA-binding proteins. This observation is likely the result of a need for RNA-binding proteins to modulate the translational control and RNA stabilization needed to regulate stage-specific protein synthesis. Interactions between mRNAs and RNA-binding proteins, such as the ELAV (embryonic lethal abnormal visual) proteins (46), CPEB (cytoplasmic polyadenylation element binding protein) (47), and TSN (12) facilitate translational regulation of mRNAs. ELAV proteins have been implicated in the posttranscriptional regulation of growth regulatory mRNAs in the cytoplasm by affecting their stability and translatability (46). CPEB binds to the cytoplasmic polyadenylation element, modulating translational repression during oocyte maturation, and is involved in mRNA localization in neuronal synapses (47). In the testis, a number of proteins, including TSN, bind to *Prm2* mRNAs during its translational repression (1, 48–50), suggesting a variety of RNA-protein complexes can be formed with germ cell mRNAs. In addition to transporting postmeiotically expressed mRNAs (12), the RNA-binding protein TSN binds to meiotic noncoding RNAs and meiotic mRNAs, such as *Dbil5*, in the nuclei of spermatocytes (51). Because TSN does not bind to the *Pgk2* mRNA (52), another mechanism to stabilize and temporally regulate its expression is needed. The need to delay translation of mRNAs, such as the *Pgk2* mRNA from early meiosis to spermiogenesis (a delay of many days), is not understood. In general, little is known of the trans-acting factors that regulate meiotic transcripts in mammals. Recent studies indicate that isoforms of polypyrimidine-tract-binding protein 2 and KH-type splicing regulatory protein are components of protein complexes that bind to the 3′UTR of *Pgk2* (53, 54) (M. Xu and N.B.H., unpublished data). The possibility that complexes containing polypyrimidine-tract-binding protein 2 or other sets of RNA-binding proteins can coordinately regulate a subset of meiotically expressed mRNAs is an attractive hypothesis that can be tested now that a population of mRNAs has been identified. Future studies will need to define consensus cis element motifs and/or shared and specific protein factors that control the expression of the translationally regulated meiotic mRNAs. Such studies would provide important insight into the mechanisms regulating translation during meiosis in the mammalian testis.

Materials and Methods

Polysomal Gradient Fractionation and Analysis. Extracts of testes from 17 day-old, 22-day-old, and adult (60–80 days old) CD-1 mice were fractionated over 10–30% sucrose gradients as described in ref. 13. To determine that the extract fractionated into RNPs and polysomes, total RNA was purified from each fraction and aliquots of purified RNAs were used for Northern blot analysis with α-^{32}P-labeled probes.

Microarray Processing. Pooled RNP and polysomal total RNAs were isolated from sucrose gradients and used for quantitative microarray analyses (Fig. 1). Polysome gradients containing three individually prepared extracts were run in triplicate, and microarrays were run for each of the 18 samples (six different samples were analyzed three times each). Affymetrix mouse GeneChip MOE430A microarrays (Affymetrix) were hybridized at the University of Pennsylvania Microarray Facility (55). In brief, 5 μg of total RNA from RNPs and polysomes was converted to first-strand cDNA by using Superscript II reverse transcriptase primed by a poly(T) oligomer that incorporated the T7 promoter. Second-strand cDNA synthesis was followed by *in vitro* transcription for linear amplification of each transcript and incorporation of biotinylated CTP and UTP. The cRNA products were fragmented to 200 nt or less, heated at 99°C for 5 min, and hybridized for 16 h at 45°C to MOE430A microarrays. The microarrays were then washed at low [6× standard saline phosphate/EDTA (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA] and high (100 mM Mes/0.1M NaCl) stringency and stained with streptavidin–phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin–phycoerythrin stain. A confocal scanner was used to collect fluorescence signal at 3-μm resolution after excitation at 570 nm. Affymetrix GCOS 1.2 software was used to analyze and quantify the hybridized arrays. Affymetrix's MAS5 algorithm (with default settings, as encoded in GCOS 1.2) was used to generate signal values and to determine present/absent/marginal flags for each probe set on each array. Probe sets flagged by MAS5 as present are described as detected.

Data and Cluster Analyses. Probe set signal values were imported to GENESPRING 7.1 (Agilent Technologies, Palo Alto, CA), where the median of each array was normalized to the median of the Affymetrix spike-in controls. This normalization was dictated by the experimental design, according to which no assumptions could be made as to the similarity of the mRNA distributions between the different gradient fractions. Normalized data and present/absent/marginal flags were then exported to Microsoft EXCEL, where within-gradient ratios were calculated between each of the nine pairs of polysome/RNP time points. Subsequent analysis was performed on the ratio data to lessen any potential gradient batch effects that might be inadequately addressed by the normalization. The ratios, geometric means of the ratios for each time point, pairwise *t* tests to detect significant differences between all three combinations of conditions, ratio differences for all three combinations, and mean signals for each of the conditions were calculated. These calculated values and the Affymetrix

flags were used to filter the data to the smaller gene sets shown in Fig. 2. Probe sets were considered present when they were scored so in at least two of the three samples. To generate the heat map, probe sets that scored as present and showed a >20% difference of the RNP-to-polysome RNA ratio in any of the pairwise comparisons (P value <0.05) were selected. Filtered probe set lists were imported to SPOTFIRE 8.1 (Spotfire, Somerville, MA), where hierarchical clustering was performed based on mean RNP-to-polysome RNA ratio [mean RNP/mean total (RNP + polysome)] for each time point. Annotations of the transcripts were updated with DAVID (http://david.niaid.nih.gov/david). All corresponding UniGene clusters were then screened for reported tissue expression by using the National Center for Biotechnology Information UniGene database.

Q-PCR Analysis of mRNA Concentration. All primers were checked by PCR to ensure that they generated single bands of the predicted size (Table 11, which is published as supporting information on the PNAS web site). PCR was performed by using the SYBR Green PCR Master Mix and the ABI 7700 thermal cycler (Applied Biosystems) at typical amplification parameters (50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min). Raw count values obtained with SDS 2.0 (Applied Biosystems) were imported into EXCEL (Microsoft) to calculate the fold changes normalized against *Gapdh* as described in ref. 56.

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